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Antimycobacterial Activity of Lichens

Vivek Kumar Gupta, Mahendra P. Darokar, Dharmendra Saikia, Anirban Pal, Atiya Fatima, and Suman P.S. Khanuja

Central Institute of Medicinal and Aromatic Plants (CSIR), Lucknow, India

Abstract

Ethanol extracts of nine lichen species, namely *Everniastrum cirrhatum* (Fr.) Hale ex Sipman (Parmeliaceae), *Flavoparmelia caperata* (L) Hale (Parmeliaceae), *Heterodermia leucomela* (L) Poelt (Physciaceae), *Lecanora flavidorufa* Hue (Lecanoraceae), *Leptogium pedicellatum* P.M. Jorg (Collemataceae), *Lobaria isidiosa* (Bory) Trevisan (Stictaceae), *Rimelia reticulata* (Taylor) Hale and Fletcher (Parmeliaceae), *Phaeophyscia hispidula* (Ach.) Essl (Physciaceae), and *Stereocaulon foliolosum* Nyl. (Stereocaulaceae), were evaluated for antimycobacteral properties against *Mycobacterium tuberculosis* $H_{37}Rv$ and $H_{37}Ra$ strains using the radiometric BACTEC method. Among the tested lichens, the virulent strain of *M. tuberculosis* $H_{37}Rv$ was found more susceptible to ethanol extract of *F. caperata* and *H. leucomela* (MIC 250 μg/mL). *E. cirrhatum*, *R. reticulata*, and *S. foliolosum* were found active at the concentration of 500 μg/mL. *L. isidiosa*, *L. pedicellatum*, *P. hispidula*, and *L. flavidorufa* did not exhibit activity at the maximum tested concentration of 1000 μg/mL.

Keywords: Antimycobacterial agents, BACTEC, lichens, *Mycobacterium tuberculosis*, radiometric assay.

Introduction

Lichens and lichen products have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world (Richardson, 1991). They produce characteristic secondary metabolites that are unique with respect to those of higher plants (Hale, 1983; Lawrey, 1986). Burkholder et al. (1944) reported for the first time the presence of antibiotic substances in lichens. The lichens *Cetraria islandica*, *Lobaria pulmonaria*, and *Cladonia* species were known for treatment of pulmonary tuberculosis (Vartia, 1973).

Tuberculosis (TB), mainly caused by *Mycobacterium tuberculosis*, is the leading killer among all infectious diseases worldwide and is responsible for more than 2 million deaths annually. India has 2% of the land area of the world and 15% of total world population but has a disproportionately high rate (30%) of the TB burden. Tuberculosis remains a serious public health problem with an annual incidence of 21 million out of which nearly 1 million are infected smear-positive pulmonary cases (WHO, 2006).

For more than 30 years, no antitubercular agents with new mechanisms of action have been developed. The recent increase in the number of drug-resistant clinical isolates of *M. tuberculosis* has created an urgent need for the discovery and development of new antituberculosis leads (Cantrell et al., 2001).

India is a rich center of lichen diversity, contributing nearly 15% of the 13,500 species of lichens so far recorded in the world (Negi, 2000). In various systems of traditional medicine worldwide, including the Indian system of medicine, these lichen species are said to effectively cure dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders, and many disorders of blood and heart (Saklani & Upreti, 1992; Lal & Upreti, 1995; Negi & Kareem, 1996; Sochting, 1999).

In the current study, we investigated nine lichen species (Table 1) of traditional importance from the Indian Himalayan flora for antibacterial activity against *M. tuberculosis*, a highly infectious microorganism, using 460TB assays.

Materials and Methods

Collection of lichens

Lichens were collected from the Narayan Asharam, Pitthoragarh District, Uttranchal, India, during April 2002. Dr. D.K. Upreti, Lichen Laboratory, National

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Address correspondence to: Dr. S.P.S Khanuja, Central Institute of Medicinal and Aromatic Plants (CSIR), Kukrail Picnic Spot Road, P. O. CIMAP, Lucknow, 226015, India. Tel.: +91-522-2359623; 2357134; Fax: 91-0522-23426666; E-mail: khanujazy@yahoo.com

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Table 1. Traditional uses of different lichen species.

Lichens	Voucher sample no.	Family	Medicinal use	References
Everniastrum cirrhatum (Fr.) Hale ex Sipman	CIMAP-12009	Parmeliaceae	Wound healing, antiseptic, bronchitis	Saklani & Upreti (1992)
Flavoparmelia caperata (L) Hale	CIMAP-12010	Parmeliaceae	Burns, fever, pain, and in the Siddha system of medicine	Saraswathy et al. (1990)
Heterodermia leucomela (L) Poelt	CIMAP-12011	Physciaceae	Wound healing	Saklani & Upreti (1992)
Lecanora flavidorufa Hue	CIMAP-12012	Lecanoraceae	Disorders of blood and heart	Sochting (1999)
Leptogium pedicellatum P.M. Jorg	CIMAP-12013	Collemataceae	No reported use	No report
Lobaria isidiosa (Bory) Trevisan	CIMAP-12014	Stictaceae	Skin diseases, chest complaints	Sochting (1999)
Phaeophyscia hispidula (Ach.) Essl	CIMAP-12015	Physciaceae	No reported use	No report
Rimelia reticulata (Taylor) Hale and Fletcher	CIMAP-12017	Parmeliaceae	Kidney disorder, veneral disease	Sochting (1999)
Stereocaulon foliolosum Nyl.	CIMAP-12018	Stereocaulaceae	Urinary trouble, blister of the tongue	Saklani & Upreti (1992)

Botanical Research Institute (CSIR), Lucknow, U.P. India, authenticated them as *Everniastrum cirrhatum* (Fr.) Hale ex Sipman (Parmeliaceae), *Flavoparmelia caperata* (L) Hale (Parmeliaceae), *Heterodermia leucomela* (L) Poelt (Physciaceae), *Lecanora flavidorufa* Hue (Lecanoraceae), *Leptogium pedicellatum* P.M. Jorg (Collemataceae), *Lobaria isidiosa* (Bory) Trevisan (Stictaceae), *Rimelia reticulata* (Taylor) Hale and Fletcher (Parmeliaceae), *Phaeophyscia hispidula* (Ach.) Essl (Physciaceae), and *Stereocaulon foliolosum* Nyl. (Stereocaulaceae). The voucher specimens were deposited at the Central Institute of Medicinal and Aromatic Plants (CIMAP) herbarium (Table 1).

Extract preparation

Lichens were air-dried at room temperature under shade. After air-drying, they were ground to fine powder in a mixer grinder. The powdered materials (1.0 g) were dipped in absolute ethanol in a percolator for 72 h at room temperature. The extracts were filtered using Whatman filter paper no. 1 and concentrated at 40°C under reduced pressure and then lyophilized to obtain fine crude extract (~5–10%). Stock solutions of 100 mg/mL were made in DMSO (Merck, India) and filter sterilized for further use in bioassays. The stock solution was stored at 4°C until use.

Mycobacterium strains

The strains of *Mycobacterium* used in this study were *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) and *M. tuberculosis* $H_{37}Ra$ (ATCC 25177), which were cultured on Löwenstein-Jansen media slant (Hi Media, India) at 37°C.

BACTEC radiometric susceptibility assay

BACTEC 460TB system (Becton-Dickinson Diagnostics Instruments Systems, Sparks, MD) was used to evaluate the antimycobacterial activity of the lichen extracts. This assay is a comparatively rapid, radiometric, drug susceptibility assay system for slow-growing *Mycobacterium* species. The BACTEC 460TB system uses BACTEC 12B medium (Becton – Dickinson), which is basically an improved Middlebrook 7H9 base enriched with additional supplements. Mycobacteria utilize a ^{14}C-labeled substrate (fatty acid, e.g., palmitic acid) present in the 12B medium, and $^{14}CO_2$ is released as a result of their metabolic activities (Siddiqi, 1996).

Preparation of inoculum

Cryopreserved *M. tuberculosis* strains $H_{37}Ra$ and $H_{37}Rv$ were taken out from a −80°C freezer and cultured on Löwenstein – Jensen medium slant. After 18–21 days of incubation, cultures were scraped from slants and transferred in 1.0 mL of BACTEC diluting fluid (Becton-Dickinson) and a complete homogenized suspension made by vortexing with glass beads (2 mm diameter). The suspension was allowed to stand for a few minutes to permit sedimentation of the bacterial clumps, if any. The turbidity of the homogenous suspension was adjusted to McFarland standard 1.0 with diluting fluid. A BACTEC 12B vial (Becton-Dickinson) was injected with 0.1 mL of this suspension. This vial was used as primary inoculum after the growth index (GI) reached a value of about 500 (approximately 1×10^6 CFU/mL).

Activity evaluation

The three stock concentrations, that is 100, 50, and 25 mg/mL of lichen extracts, were prepared by dissolving in DMSO. From these stocks, 40 µL was transferred into BACTEC 12B vials containing 4.0 mL of medium so that the final concentration of test compounds became 1000, 500, and 250 µg/mL.

Bacterial suspension (0.1 mL) from the primary inoculum culture vial (GI ~ 500) was injected into test vials using 1.0 mL insulin syringe. To comply with 1% proportion method (Tarrand & Groschel, 1985), 0.1 mL of primary inoculum was added to 9.9 mL BACTEC diluting fluid to obtain 1:100 dilutions. From this, 0.1 mL was injected into two 12B vials containing 4.0 mL medium along with 40 μL of DMSO that was used as controls.

Vials were incubated at 37°C, and the GI was recorded every 24 h in a BACTEC 460TB instrument. Once the GI of the control vial (1:100) reached 30, then the GI values of the test vials were compared with that of control vials based on difference in growth (ΔGI). The result was interpreted as follows: If the difference (called the ΔGI) of current GI from previous day GI in the case of drug-containing vials was lower than the ΔGI of 1:100 control vial for the same period, then the test compound was termed as active. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which the ΔGI in the treated vial was less than that of the control.

Results

The ethanol extracts of nine lichens (Table 1) were tested against two strains of *M. tuberculosis*, $H_{37}Ra$ and $H_{37}Rv$, through the BACTEC radiometric assay. Among these, five lichens, namely, *E. cirrhatum*, *F. caperata*, *H. leucomela*, *R. reticulata*, and *S. foliolosum* were found active against *M. tuberculosis*. The strains $H_{37}Ra$ and $H_{37}Rv$ were found susceptible to these four lichens at a concentration range of 250 to 500 μg/mL after at least 7 days of inhibition with ΔGI unit lesser than that of control (Table 2, Fig. 1). The extract of *F. caperata* was found most active among the lichen species used with the MIC at 250 μg/mL.

The virulent strain $H_{37}Rv$ was found more susceptible to ethanol extract of lichen *H. leucomela* (MIC 250 μg/mL). *E. cirrhatum*, *R. reticulata,* and *S. foliolosum* exhibited MIC at 500 μg/mL against both the strains, whereas *L. isidiosa*, *L. pedicellatum*, *P. hispidula*, and *L. flavidorufa* did not exhibit activity even at maximum tested concentration of 1000 μg/mL. Rifampicin and isoniazid, the front-line antitubercular drugs, were used as positive control.

Table 2. Antibacterial activity of lichen extracts against *Mycobacterium tuberculosis* strains.

	MIC (μg/mL)	
Lichens	*M. tuberculosis* $H_{37}Rv$	*M. tuberculosis* $H_{37}Ra$
Everniastrum cirrhatum	500	500
Flavoparmelia caperata	250	250
Heterodermia leucomela	250	500
Lecanora flavidorufa	>1000	>1000
Leptogium pedicellatum	>1000	>1000
Lobaria isidiosa	>1000	>1000
Phaeophyscia hispidula	>1000	>1000
Rimelia reticulata	500	500
Stereocaulon foliolosum	500	500
Antibiotics		
Rifampicin	0.25	0.5
Isoniazid	0.1	0.1

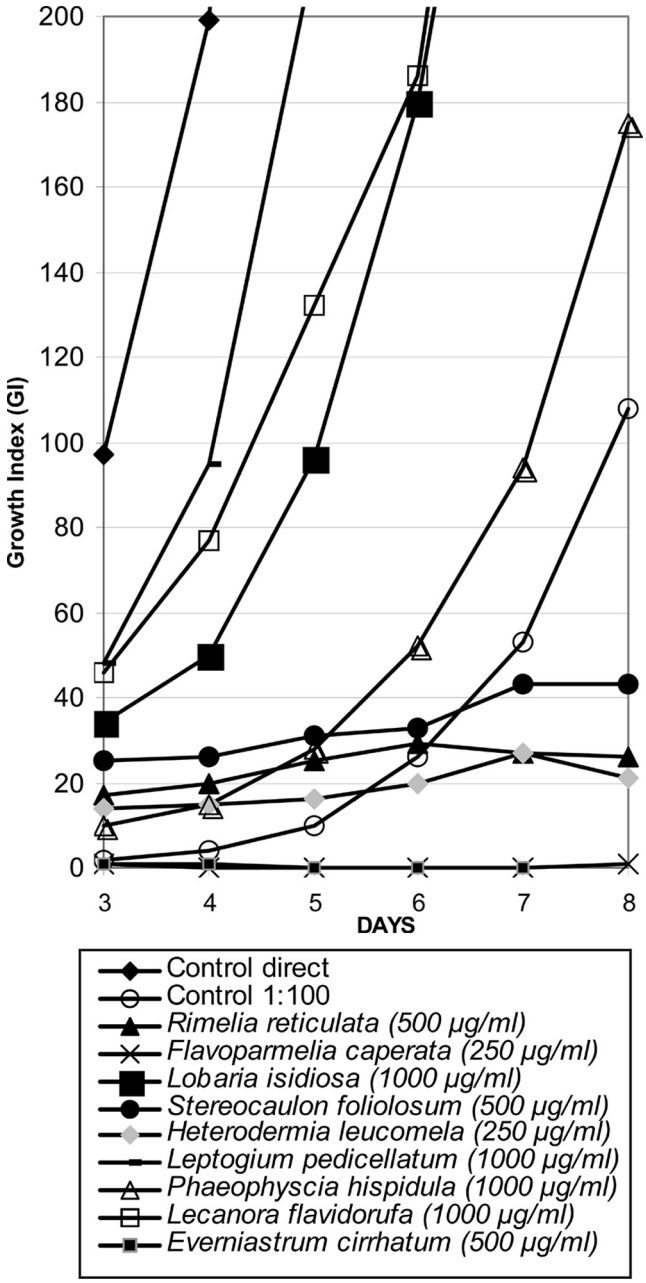

Figure 1. Growth index (GI) values of *M. tuberculosis* $H_{37}Rv$ in presence of lichen extract obtained from BACTEC assay.

Discussion

Despite intense efforts to control this disease, tuberculosis remains an expanding global health crisis claiming

2 million to 3 million human lives every year. Therefore, today tuberculosis as a disease to be managed is attracting the attention of researchers globally. With the emergence of drug-resistant strains of *M. tuberculosis*, the need to search for new antituberculosis drugs has become a necessity. Using a BACTEC 460TB assay, we were able to detect the potential of lichens as growth inhibitors of *M. tuberculosis*.

Numerous lichens were screened for antibacterial activity in the beginning of the antibiotic era in the 1950s (Klosa, 1953). Several lichen metabolites were found to be active against Gram-positive organisms (Lauterwein et al., 1995).

The antimycobacterial activity of lichen compounds was reported by Ingolfsdottir et al. (1998) against nontubercular species of *Mycobacterium*. These compounds exhibited very high minimum-inhibitory-concentration MICs against non-pathogenic *Mycobacterium aurum* for example, atranorin from *Stereocaulon alpinum* (250 μg/mL), lobaric acid from *S. alpinum* (125 μg/mL), salazinic acid from *Parmelia saxatilis* (250 μg/mL), and (+)-protolichesterinic acid from *Cetraria islandica* (250 μg/mL), except usnic acid from *Cladonia arbuscula*, which was effective at lower concentration (32 μg/mL).

Although the antibacterial activity of some lichen species has been reported against nonpathogenic and nontubercular mycobacteria, no report was found during a literature search against *M. tuberculosis*. The lichen species used in this study have not been explored earlier for this activity. The radiometric BACTEC assay appears to be more accurate, efficient, and high throughput compared with normal assays where observations are recorded based on visible growth (Chung et al., 1995; Adeniyi et al., 2004).

The results of this study indicated that four out of the nine lichen species commonly used in folk medicine were active against *M. tuberculosis*. Among four lichens, extracts of *Flavoparmelia caperata* and *Heterodermia leucomela* exhibited significant activity (MIC 250 μg/mL) at extract level against the virulent strain $H_{37}Rv$ using BACTEC assay. Based on our observations and earlier reports, it appears that the molecule(s) responsible for antibacterial activity against tubercular bacilli in active lichen species of our study may be novel with different mode of action. Our laboratory is further investigating bioactive constituents of active lichen species through bioactivity-guided fractionation and also evaluating these against drug-resistant strains of *M. tuberculosis*.

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